SELECTED MECHANISTIC ASPECTS OF VIRAL INACTIVATION BY PERACETIC ACID

by

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Abstract

Chlorination is the most widely used disinfection process in water treatment. However, chlorination generates disinfection by-products (DBPs) that are potentially carcinogenic. Peracetic acid (PAA) is considered a strong alternative disinfectant, as previous studies have demonstrated its ability to inactivate viral pathogens. However, the underlying mechanism is understudied with respect to the virus structure components that are susceptible to oxidation via PAA.

Racemic mixtures of 20 basic amino acids, plus cystine, and 4 nucleotides were subjected as the oxidation targets for PAA, at a molarity ratio of 1:5 (PAA:target). Two-hour PAA decay tests were conducted in phosphate buffer (1 mM, pH 7) in triplicate and PAA was measured spectrophotometrically. To exclude the possible impact of hydrogen peroxide present in commercial PAA solutions, additional experiments were performed with pure hydrogen peroxide under similar conditions and analyzed using ferric thiocyanate spectrophotometry.

Results showed that amino acids with sulfur-containing R groups (cysteine and methionine) reacted with PAA in less than two minutes. The consumption of PAA by

tryptophan and cystine followed pseudo-first-order kinetics, and the rate constants were determined to be 1.4×10^{-3} min^{-1} and 1.9×10^{-2} min^{-1}, respectively. However, the majority of amino acids (17 out of 21) and all RNA nucleotides (4 out of 4) did not show active reaction with PAA, when compared with controls. In addition, H_2O_2 was demonstrated to be less oxidative than PAA. The only target that reacted with H_2O_2 was cysteine, with consumption rate constant of 4.3×10^{-3} min^{-1}.

These results indicated that viruses with capsid structures containing higher proportions of cysteine, methionine and tryptophan could be more vulnerable to inactivation via PAA oxidation, while nucleic acids play a less important role in PAA disinfection. Susceptible amino acid abundances in capsids of MS2 bacteriophage and murine norovirus were proven to be close to each other. The resistance of MS2 bacteriophage to PAA disinfection observed in previous studies may partially be attributed to MS2 capsid AA composition.

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Chapter 1

Introduction

1.1 Thesis Statement

A comprehensive evaluation of the disinfectant - peracetic acid (PAA) consumption by amino acids and nucleotides, which are the key viral structural and genetic components of viruses was conducted.

1.2 Global Water Use and Reuse

As the most important resource on earth, water is essential to life and all activities. Freshwater that can be directly used for human uses is limited due to increasing water demands accompanied with rapid population growth. In recent years, water scarcity has been exacerbated by climate change. Water quality is declining because of flooding and sea level rise caused by greenhouse effects. It has been estimated that nearly half of the world's population will be living in areas that are short of water by 2050.[1] More freshwater

is being withdrawn to meet the enormous demands for living and production of human products and services. At the same time, overuse of water can result in harm to ecosystems, especially those depending on shallow groundwater or perennial streams.[2]

To combat these problems, water utilities are incorporating wastewater reclamation and reuse as an alternative source of drinking water. In conventional municipal water systems, groundwater or surface water is withdrawn for water supply and distributed for use after meeting relevant standards with treatment. Wastewater is discharged into surface water after specific treatment to remove organic compounds and pollutants. Water reuse refers to the use of treated wastewater for beneficial purposes, such as drinking, irrigation, and industrial uses.[3] Reuse can provide an opportunity for communities facing water shortage to significantly expand their limited water supply.

There are two main types of water reuse: potable reuse and non-potable reuse.[3] The purpose of potable water reuse is for drinking and other municipal uses, while non-potable reuse refers to wastewater treated for agricultural, industrial, or recreational purposes. Potable reuse can also be categorized into direct and indirect reuse. In direct reuse, treated wastewater is directly used as the source water for drinking water treatment plants. Both carbon footprint and cost can be reduced with less procedures for discharge and withdrawal when using direct potable reuse. In contrast, indirect reuse can be defined as point-source discharge of treated wastewater into groundwater or surface water, where there is an environmental "buffer" stage.[4] The sedimentation and filtration from the aquifer and natural water bodies purify the water, and the water is withdrawn and transported to drinking water facilities for further treatment prior to distribution. This method of water reuse receives more positive public perception, and perceived safety concerns are addressed by natural purification.

1.3 Public Health Concerns with Water-borne Microorganisms

Municipal wastewater typically contains a wide range of chemical and microbial contaminants. Bacteria, viruses and parasites are present in wastewater largely due to high levels of fecal contamination. Since 1970, several species of microorganisms have been confirmed as pathogens by World Health Organization.[5]

Water-borne enteric bacteria can pose substantial health risks to humans. *Escerichia coli* is one of the most common causes of bacterial diarrhea. Similarly, the genus *Salmonella* is also one of the most common pathogens found in municipal wastewater, which contains a wide variety of species that are harmful to humans and animals. The most severe form of salmonellosis is typhoid fever. In addition, *Shigella* can produce bacillary dysentery or shigellosis.[6]

Viral pathogens, such as rotaviruses, noroviruses and adenoviruses present concerns in water reclamation and reuse schemes as they are persistent throughout treatment processes due to small size, resistance to conventional disinfection and high infectiousness even at low doses.[3] Water-borne viruses are the main cause of gastroenteritis.[6]

There also have been outbreaks associated with protozoa contamination in water.[7] As widespread parasites in nature, *Cryptosporidium* and *Giardia* can also occur in wastewater and these protozoa are pathogenic. In particular, *Cryptosporidium* oocysts are difficult to remove from water through conventional processes.[8]

To sum up, microorganisms in reclaimed water can cause a number of illnesses and a major goal of disinfection is to inactivate pathogens prior to discharge into the environment or downstream reuse. Therefore, wastewater effluent must be treated

appropriately in wastewater reuse to ensure efficient downstream treatment in reuse cycles and to minimize public health risks. Strong chemical disinfection processes are especially needed for potable use, even in systems that include additional advanced treatment.

1.4 Common Disinfection Techniques

1.4.1 Chlorine

Chlorine is the most widely used disinfectant in current water disinfection systems due to its low cost and efficiency in inactivating a majority of microorganisms.[9] However, the formation of disinfection by-products (DBPs) related with chlorination has become an emerging health concern. More than 600 DBPs have been identified till now.[10] Of these, trihalomethanes (THMs) and haloacetic acids (HAAs) are considered the most concerning due to their potential to be carcinogenic, mutagenic and genotoxic. They are currently under the regulation of US Environmental Protection Agency (THMs and HAAs) and European Union (THMs).[11] The residual free chlorine is also toxic to aquatic ecosystems if not appropriately inactivated before discharge. Additionally, chlorine can be a safety concern during transport and storage.[12] Therefore, there is an increasing need for alternative disinfection methods that can both provide efficient microorganism inactivation and be environmentally friendly.[13]

1.4.2 Ozone

Ozone is also a strong biocide used for wastewater disinfection. It is more effective than chlorine in inactivating viruses, and a relatively shorter contact time is needed (half-life in water approximately 10 to 30 minutes).[14] Ozone is generally produced onsite because it is

unstable and decomposes to elemental oxygen in a short period of time after generation. However, ozonation requires more complex equipment, compared to chlorination.[15] Therefore, the cost of equipment is considerable when applied in large-size operations and the energy demands can be intensive.

1.4.3 Ultraviolet Irradiation

Ultraviolet (UV) irradiation was considered a "clean" disinfection technique, but it has the potential to produce aldehydes.[16] Also, its efficiency highly depends on the quality of water to be treated. UV proved to be effective in effluent with low turbidity and relatively less suspended solids.[17] In addition, the method of UV disinfection is rather expensive and energy consuming, and requires regular cleaning.[13]

1.5 Peracetic Acid as a Disinfectant

Peracetic acid, CH_3COOOH, is a weak acid and peroxygen that is highly water soluble and unstable. Therefore, commercial PAA is available in the quaternary equilibrium mixture with acetic acid (CH_3COOH), hydrogen peroxide (H_2O_2) and water. PAA contains an additional oxygen atom bound to the molecule of acetic acid, which provides a high oxidation potential (1.960 V), which is greater than that of hydrogen peroxide (1.776 V), chlorine (1.358 V in gas) and chlorine dioxide (1.277 V).[18,19] The decomposition products of PAA include acetic acid, water and oxygen, which are not harmful to humans. The antimicrobial property of PAA was first reported by Freer and Novy (1902).[20] It has been applied in food-processing, beverage and diary industries, as well as medical instrument sterilization.[19,21,22] PAA has also begun to be implemented as a disinfection treatment option for wastewater, drinking water and even water reuse as a promising alternative to

chlorine, because of its effectiveness of wide-spectrum antimicrobial activities and limited formation of harmful disinfection by-products.

The method to detect low concentrations of PAA was adapted from DPD (N, N-diethyl-p-phenylenediamine) colorimetric method for total chlorine residual determined by US EPA Method #330.5.[23] In general, an excess of potassium iodide (KI) is oxidized by PAA into iodine (I_2). I_2 then reacts with DPD to form a pink color, for which the absorbance is linearly proportional to PAA concentration.[24] The existence of H_2O_2 can potentially interfere with PAA analysis, due to its ability to oxidize iodide. However, it has been proven that H_2O_2 reaction requires the addition of catalysts and a longer reaction time compared to PAA.[25] Therefore, the interference is negligible.[26] This method has been widely discussed and applied in previous studies.[27-29] Importantly, the DPD method needs to be performed under optimal pH condition (4-6.5) to measure PAA, as a significant decrease in absorbance can be observed outside of this range.[30]

1.5.1 Effectiveness of PAA Disinfection

Fast and effective inactivation of various bacteria, fungi and spores in different water matrices by PAA has been demonstrated by previous studies.[29,31-34] Antonelli et al. (2006) suggests that PAA residues became negligible in 5 to 11 hours, and there was no significant re-growth of coliform bacteria after 29 hours of disinfection.[35]

In 1999, the US EPA suggested that PAA could be evaluated as a disinfectant for combined sewer overflows (CSOs).[36] McFadden et al. (2017) confirmed that PAA was an effective disinfectant for CSOs containing suspended solid particles in different sizes. Hypochlorite was proven to be less efficient than PAA, as it required much higher concentration-time (CT) to initiate effective disinfection and more easily affected by particle size.[37] This is in agreement with Lefevre et al. (1992) that PAA was insensitive to

the presence of suspended solids (up to 100 mg/L).[33]

PAA has also shown capability to inactivate viruses, as bacteriophages in secondary and tertiary effluent have been effectively removed at a dose of 1-5 mg/L PAA and a contact time of 60 min.[33,38] In Dunkin et al. (2017),) the 1-, 2- and 3-$\log_{10}$ model CT values for murine norovirus (MNV) reduction in municipal wastewater by PAA disinfection were predicted to be 32, 47 and 69 mg-min/L, respectively.[24]

1.5.2 Mechanisms of PAA Inactivation

PAA has a much stronger antimicrobial capacity than its decomposition products, hydrogen peroxide and acetic acid.[31] The inactivation mechanism of microorganisms by PAA is still not completely understood. Some studies have proposed that the disinfection mechanisms of PAA is associated with the release of reactive oxygen species (ROS), including hydroxyl (HO·), alkoxyl (RO·), hydroperoxyl (HO_2·) radicals and superoxide (O_2·), as well as organic radicals including acetyl (CH_3COO·) and methyl (CH_3·) radicals. These strongly oxidative species disrupt thiol groups (-SH) and disulfide bonds (S-S) within enzymes, proteins and other metabolites, enabling the dislocation of chemiosmotic function and transport across cell membranes through dislocation or rupture of cell walls.[19,33] Once inside the cell, PAA can oxidize essential enzymes, impairing essential biochemical pathways, active transport and intracellular solute levels.[39] In addition, PAA was suggested to inactivate catalase, an enzyme known to detoxify hydroxyl radicals. The ovicidal and sporicidal properties of PAA may be explained by its effects as a protein denaturant.[39,40] The organic radicals produced by PAA have longer half-lives and higher effectiveness in antimicrobial action than hydroxyl radicals.[13,40]

Flores et al. (2014) indicated that the efficiency of bacterial inactivation by commercial PAA was higher than PAA or hydrogen peroxide acting alone. A potentiating

synergetic effect was proposed, as the effect of the mixture was proven even greater than the sum of two individual oxidants. This study also suggested a mechanism of radical chain reactions to explain the rapid kinetics of PAA disinfection.[41]

1.5.3 By-products from PAA Disinfection

DBPs formed by PAA disinfection were reported mostly as carboxylic acids that were not recognized as mutagenic or genotoxic, and no carcinogenic/mutagenic halogenated DBPs were detected.[42-44] Carboxylic acids are commonly produced from drinking water disinfection by other agents, such as ozone, chlorine and chloramines, whereas halogenated by-products are typically found in chlorine-based water disinfection.[42,45] A recent study systematically investigated the formation of THMs and HAAs during drinking water disinfection by PAA, indicating that the ratio of hydrogen peroxide and PAA concentration in commercial PAA solutions had a significant effect on the formation of these potentially concerning by-products. No detectable levels of both by-products were observed when PAA concentration was lower than H_2O_2, while low levels of I-THMs and I-HAAs were detected when PAA concentration was higher than H_2O_2.[46] In general, the level, variety and hazard of DBPs formed by PAA disinfection are not comparable to those formed by chlorination or ozonation.

The adverse effects observed on different indicator organisms have basically been attributed to the residues of disinfectant. Antonelli et al. (2009) pointed out that the possible influence of PAA residuals on biological processes in receiving aquatic environment cannot be completely ignored.[47] On the other hand, the rapid degradation of PAA related with water matrix composition was a benefit from the perspective of reducing exposure and environmental impact. Moreover, the low likeliness of bioaccumulation of PAA, hydrogen peroxide and acetic acid in aquatic organisms was affirmed by their

octanol-water partition coefficients (K_{OW}) (0.3, 0.4 and 0.68, respectively).[45]

1.5.4 Challenges for the Use of PAA Disinfection

The major drawbacks related with PAA disinfection include strong dependence on the water quality of effluent, hindered inactivation activities in alkaline environments, and limited efficiency against resistant microorganisms.[18,31] Pilot experiments were conducted in wastewater treatment plants by Liberti and Notarnicola (1994), demonstrating that low doses (1-5 mg/L) of PAA were insufficient for municipal wastewater disinfection for agricultural reuse under a standard of 2 CFU/100 mL total Coliform, as the existence of organics or oxidizable compounds consumed the disinfectant rapidly.[48] Domínguez-Henao et al. (2018) have confirmed that the initial consumption of disinfectant in PAA wastewater disinfection were influenced by organic compounds, mainly proteins, while the rate constant was determined by inorganics.[49] It was also reported that the efficiency of PAA was decreased at pH above 7.5, and a significant decrease was demonstrated at pH 9.[19,37] Although Dunkin et al. (2017) indicated the reduction of murine norovirus by PAA disinfection,[24] PAA has been proven to be basically ineffective towards *Giardia* and *Cryptosporidium*.[48]

1.6 Research Objectives and Significance

Previous studies have demonstrated the ability of PAA to inactivate murine norovirus, while a less significant efficiency was observed when inactivating MS2 bacteriophage.[24,50] It was suggested that the inactivation of bacteriophages might occur by damaging some specific viral surface structure, such as the protein coat or the attachment sites needed for infection of host cells.[50]

This study aims to explore underlying mechanisms of viral inactivation via PAA, specifically with respect to virus structural and genomic components that are susceptible to PAA oxidation. A comprehensive evaluation of reactivity of amino acids and RNA nucleotides with PAA was performed under practical operational environments. The findings will provide a better understanding for PAA as an alternative to chlorine, which can potentially be effective in inactivating viruses in wastewater.

Chapter 2

Materials and Methods

2.1 Reagents

In this study, DL-racemic mixtures of 20 basic amino acids plus cystine and 4 RNA nucleotides were chosen as targets for consumption of PAA. All amino acids were purchased from Sigma Aldrich (St Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA). Adenosine monophosphate (AMP) and cytidine monophosphate (CMP) were purchased from Sigma Aldrich (St Louis, MO, USA), and guanosine monophosphate (GMP) and uridine monophosphate (UMP) were purchased from OChem (Chicago, IL, USA). All chemicals were at a purity level of >97%. Solutions were prepared with reagent deionized water generated from a Milli-Q advantage water purification system (Millipore, Billerica, MA, USA). Working stock solutions were prepared at 5 g/L by diluting the commercial stock. PAA working stock was prepared every week from commercial PAA solution (21% PAA, 26% H_2O_2, w/w; PeroxyChem, Philadelphia, PA, USA). Hydrogen peroxide working stock was freshly made every day from the commercial solution (35% H_2O_2 in water, w/w; Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Analytical Methods

2.2.1 Titration for PAA and Hydrogen Peroxide

Concentrations of PAA and H_2O_2 in commercial PAA solution were determined by iodometric titration, as previously described by Domínguez-Henao et al. (2018).[30] Briefly, PAA and H_2O_2 oxidize iodide into iodine under acidic condition, which is then titrated by sodium thiosulfate ($Na_2S_2O_3$) solution of known concentration with the existence of starch indicator (Aqua Solutions, Deep park, TX, USA). Due to the potential of H_2O_2 to react with KI, bovine catalase (2000-5000 units/mg; Sigma Aldrich, St Louis, MO, USA) was added for quenching. The concentration of PAA can be derived from the volume of consumed $Na_2S_2O_3$ standardized solution. To measure the sum molarity of PAA and H_2O_2, ammonium molybdate [$(NH_4)_2MoO_4$; HACH Company, Loveland, CO, USA] was used as a catalyst without quenching with catalase. The molarity of H_2O_2 was calculated by subtracting PAA molarity from the sum. The reactions are as illustrated in the following:

$$CH_3COOOH + 2KI + H_2SO_4 \rightarrow CH_3COOH + I_2 + K_2SO_4 + H_2O \quad (1)$$

$$H_2O_2 + 2KI + H_2SO_4 \xrightarrow{(NH_4)_2MoO_4} I_2 + K_2SO_4 + 2H_2O \quad (2)$$

$$I_2 + 2Na_2S_2O_3 \rightarrow 2NaI + Na_2S_4O_6 \quad (3)$$

Titration for commercial H_2O_2 solution was performed with standardized potassium permanganate ($KMnO_4$) solution. H_2O_2 can be oxidized by $KMnO_4$ with an excess of sulfuric acid (H_2SO_4), according to Eq. (4):

$$2KMnO_4 + 5H_2O_2 + 6H_2SO_4 \rightarrow 2MnSO_4 + 5O_2 + 8H_2O \quad (4)$$

2.2.2 Colorimetric Analysis for PAA and Hydrogen Peroxide

The DPD method was performed using HACH DPD reagent powder pillows for total

chlorine (HACH Company, Loveland, CO, USA). After treating the sample with the powder pillow, the absorbance was measured at a wavelength of 530 nm using spectrophotometer HACH DR6000. The concentration of PAA was then determined via the molecular weight ratio of chlorine to PAA (1:1.07).

The ferric thiocyanate method was used to analyze H_2O_2, as previously described by Boltz and Howell (1978).[51] Briefly, H_2O_2 oxidizes ferrous iron to the ferric state under acidic condition, which then forms a red complex with ammonium thiocyanate (NH_4SCN) and can be measured at a wavelength of 470 nm. The method was implemented using commercially-available test kits (CHEMetrics, Midland, VA, USA).

2.3 Experimental Design

To evaluate the optimal concentration and pH of phosphate buffer (PB) for measuring PAA, commercially available PB (130 mM; Thermo Fisher Scientific, Waltham, MA, USA) and laboratory generated PB (100 mM, 10 mM, 1 mM and 0.1 mM) at pH 7 and 6 were used as reaction matrices. One-hour PAA analysis experiments were performed in different PBs. Samples were taken and analyzed at 2, 30 and 60 min. In addition, sodium chloride (NaCl) and potassium chloride (KCl) solutions were employed to exclude the potential effect of cations. Selected experiments were also performed in sealed vials in order to evaluate the potential volatilization of PAA that might occur under open reaction system and magnetic stirring conditions.

The PAA experiments were performed in 250-mL glass beakers at room temperature. As determined in preliminary experiments, 1 mM PB at pH 7 was used to dissolve the target and maintain the pH of the reaction system. For those amino acids and nucleotides having a strong impact on solution pH, including aspartic acid (ASP),

glutamic acid (GLU), lysine (LYS), arginine (ARG), AMP and CMP, sulfuric acid and sodium hydroxide solution (0.1 N) was added to adjust pH to 7 before the experiment was started. PAA working stock was then spiked into the solution containing 200 μM target, ensuring the PAA/target molarity ratio at 1:5 and the reaction solution was mixed by gentle magnetic stirring for 120 minutes. Aliquot (10 mL) samples were collected and PAA was measured at specific ime points: 2, 5, 10, 30, 60, 120 min. As controls, PAA in phosphate buffer was measured in the absence of targets. Measurements were conducted using DPD method as specified in Section 2.2.2.

To exclude the potential impact of H_2O_2 on PAA consumption, experiments were performed using H_2O_2 as the only oxidant, with the molarity adjusted to 110 μM in consistency with the molar ratio of PAA/H_2O_2 (1:2.75) in PAA commercial stock. All settings of H_2O_2 experiments were kept the same with PAA experiments. Measurements were conducted using the ferric thiocyanate method as specified in Section 2.2.2. All experiments for each target were conducted in triplicate.

2.4 Statistical Analysis and Viral Capsid Component Modeling

Disinfectant consumption rates (k', min^{-1}) were calculated by regressing the observed residuals at different reaction time points, as illustrated in eq. (5).

$$\frac{d[PAA]}{dt} = -k'[PAA] \qquad (5)$$

Where [PAA] is the concentration of PAA. Statistical analysis was performed using RStudio software (Version 1.1.463; Boston, MA, USA).

The amino acid components of viral capsids were obtained from European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) online database[52] and abundances of selected amino acids were accordingly calculated. The products of amino acid abundances with corresponding PAA consumption rate estimated before were summed to predict the reactivity of PAA with different viral capsids.[53]

Chapter 3

Results and Discussion

3.1 pH and Concentration Range-Finding for the Use of Commercially-available PAA Analysis Reagents

Prior to consumption experiments, concentrations of commercial PAA (21% PAA, 26% H_2O_2, w/w) and commercial H_2O_2 (35% H_2O_2, w/w) were confirmed via titration as specified in Section 2.2.1. The results were shown in Table 1.

When performing initial control experiments using HACH reagent powder pillows for measuring PAA in phosphate buffer, it was observed that the pH and concentration of PB were important parameters that needed to be optimized. Therefore, preliminary experiments on pH and concentration range finding were performed. The results indicated that high concentration of PB could interfere with HACH DPD method for PAA analysis, and that there was a decrease in concentration over 1 hour when 10mM PB was used. These experiments indicated 1 mM PB was sufficient to maintain the desired pH while dissolving 3 ppm PAA and most targets, as well as being the most appropriate conditions to perform the HACH DPD method in PAA disinfection experiments (Table 2). Other studies have reported that for functional DPD methods the sample pH must be

adjusted within a certain range (4–6.5).[30] If this was not done, a significant decrease in expected measurements would be observed and that when the concentration of PB was too high, the sample became "over-buffered" so that the pH could not be adjusted to the optimal analysis range. The previous finding in pH range was also confirmed by this study, as the analysis results at pH 6 were found to be closer to expected concentration than pH 7. However, when considering the pH of wastewater under practical conditions, pH 7 was chosen instead of pH 6. Also, the possible impacts of cations (K^+ and Na^+) in PB and open reaction system have been excluded through comparison experiments (Tables 3).

3.2 Consumption of PAA by Amino Acid and Nucleotides

Among the 20 basic amino acids, cysteine (CYS), methionine (MET) and tryptophan (TRP) were proven to be the most reactive with PAA (Table 4). These findings are consistent with previous reports on PAA-induced oxidation on dairy proteins.[54] PAA was almost consumed completely by CYS in less than 2 minutes. The measurements of PAA after 2-min reaction with CYS were determined to be lower than the method detection limit (0.10 ppm). Therefore, the PAA consumption percentage by CYS should be larger than 96.7%. As for the other amino acid with side chain of sulfur group, MET consumed 90.1% of PAA in 2 minutes, and the consumption increased slightly in the rest of 120 min. TRP and cystine were also found to be actively reacting with PAA, with 23.0% and 89.1% consumption during reaction time of 120 min. Little consumption of PAA was observed for 17 out of 21 amino acids under employed reaction condition compared to controls ($[Target]_0$=200 μM, $[PAA]_0$=40 μM, pH=7). It was also shown that 4 RNA nucleotides all had minimal oxidation by PAA (Table 5).

The results confirmed the modeling prediction in Du et al. (2018) that TRP could

be a potential substrate for PAA oxidation, while leucine (LEU) and threonine (THR) may show less reactivity.[55] A previous study conducted on consumption of monochloramine by nucleotides and amino acids under the same reaction condition (molar ratio 1:5, pH 7) revealed that CYS, MET, TRP and cystine were the most reactive amino acids, which consumed 100% of monochloramine in 2 minutes.[56] Considering the relatively lower reaction rate of PAA with TRP and cystine, the ability of oxidation for PAA was proven to be weaker than monochloramine. Besides, histidine (HIS) and lysine (LYS) were reported to be easily oxidized by commercial PAA formulation. In Finnagen et al. (2010), LYS was oxidized by commercial PAA into *N*-(hexanoyl)lysine at a molar ratio of 1:10 (PAA:amino acid), and HIS was oxidized into 2' oxo-histidine (molar ratio 1:10) and di-histidine (molar ratio 2:1).[57] The reactivity of HIS was in agreement with Du et al. (2018) which was performed at a molar ratio from 13:1 to 130:1.[55] However, it was not observed in this study. It could be attributed to the different ratios of PAA to amino acids.

3.3 Consumption of H_2O_2 by Amino Acid and Nucleotides

Finnegan et al. (2010) indicated that liquid H_2O_2 can actively react with some amino acids.[57] Since H2O2 is a major component of commercial PAA solutions and also an oxidizing agent itself, experiments were performed using only H_2O_2 at the concentration of H_2O_2 present in PAA to validate that PAA consumption was mainly responsible for target oxidation (as described in Section 2.3). According to the results (Table 5), CYS was the only target that showed active consumption of H2O2, 42.8% in 120 min. The reaction rate was much lower, however, than that of CYS with PAA. All nucleotides and other amino acids did not display consumption with H_2O_2 under the employed reaction condition ($[Target]_0$=200 μM, $[H_2O_2]_0$=110 μM, pH=7). To conclude, the oxidation of targets was

driven by PAA rather than H_2O_2. It is in agreement with previous findings that the effect of background H_2O_2 on PAA reaction with amino acids were negligible.[55] These experiments also demonstrated the effectiveness and stability of commercially available test kits for H_2O_2.

3.4 Reaction Kinetics Modeling

The reaction of CYS and MET with PAA occurred too rapidly to evaluate the kinetics. For TRP and cystine, the concentration of PAA was expressed as the natural log fraction of the initial concentration, and linear regression plot indicated pseudo-first-order kinetics (Figure 1). The consumption rate of PAA with TRP at pH 7 and a 1:5 molar ratio is 1.4×10^{-3} min^{-1}, and with cystine is 1.9×10^{-2} min^{-1}. Despite the finding that PAA consumption rate for cystine is larger than that for cystine, TRP reacted immediately with PAA in the first 2 min, while significant consumption by cystine was only observed following 5 min. The consumption rates of PAA towards CYS and MET were estimated to be at least larger than 1.7 min^{-1} and 1.2 min^{-1}, respectively. In addition, the reaction of H2O2 with CYS also followed pseudo-first-order kinetics (Figure 2). The consumption rate of H_2O_2 with CYS is 4.3×10^{-3} min^{-1}.

3.5 Evaluation of Sulfur Amino Acids

In this study, the first three amino acids that consumed PAA most rapidly were all sulfur amino acids, the high reactivity of which with various oxidants was already confirmed by early studies. As indicated in early studies, thiol group of CYS could be oxidized into sulphenic acid (or CYS dimer), sulphinic acid and sulphonic acid by PAA and sulphenic

acid and sulphinic acid by H_2O_2 based on different ratios.[55,57] PAA could also oxidize the thioether group of MET to sulphoxide and sulphonic acid. And TRP was found to be oxidized to N-formyl kynurenine by PAA.[55,57] In this study, cystine was also evaluated, which was formed by oxidation of two molecules of cysteine, with disulfide bond instead of thiol group. Comparatively, the reaction rate of cystine was not as fast as CYS and MET. It was indicated that the reactivity of disulfide bond was lower than thiol group and thioether group.

3.6 Evaluation of Mechanisms of Selected Viral Inactivation by PAA

Integrity of both the capsid and genome is essential to maintain the infectious capacity of viruses.[58] Diversified viral components can be targeted by different disinfectants. In this study, the low reactivity of all RNA nucleotides with PAA indicated that capsid was the major target for viral disinfection by PAA, rather than the genome. Besides, PAA was generally thought to oxidize side chains of proteins.[57] In respect to typical RNA viruses, previous study has confirmed that PAA was unable to provide an inactivation of 1-log PFU/mL for MS2 bacteriophage even the concentration time value is higher than 800 mg-min/L. Nevertheless, an over 4-log PFU/mL of inactivation for murine norovirus by PAA was observed at concentration time values lower than 100 mg-min/L.[24]

Methods were established to predict relative reaction rates of protein target, as specified in Section 2.4. CYS, MET and TRP were identified as the amino acids that could actively react with PAA. Only these 3 amino acids in capsids were considered as reaction sites for evaluating the reactivity of virus capsids. Since the consumption rates of PAA for CYS and MET were not available, the estimated minimum rates were employed (1.7 min^{-1}

and 1.2 min^{-1}, respectively). The abundances of selected amino acids in viral capsids for MS2 bacteriophage and murine norovirus were rather close to each other (Table 6). It was also the same with the predicted reaction rates for both viral capsids, 0.045 min^{-1} for MS2 bacteriophage and 0.047 min^{-1} for murine norovirus. However, it is worth noting that the total amino acid number of murine norovirus capsid (95580 in total) is much higher than that of MS2 bacteriophage capsid (23220 in total). This fact could help to explain some of the differences in inactivation rates between both viruses.

On the other hand, it was indicated that structural data were as essential as composition data to describe the reactivity of virus components with disinfectants.[58] Capsid structures and locations of selected amino acids in capsids of MS2 bacteriophage (Figure 3) and murine norovirus (Figure 4) were visualized by PyMOL Molecular Graphics System (Version 2.3.1; Schrödinger, LLC). From the perspective figures, no obvious differences could be observed, for the residues of CYS, MET and TRP were mostly buried in the spatial structure of the proteins, rather than sticking out on the capsids and easily accessed by PAA. Based on these results, it was suggested that the locations of the susceptible amino acids in capsid of murine norovirus were associated with key binding sites that could be important to fundamental functions of virus, such as host attachment. The reaction between PAA and the side chains of these amino acids brought about the damage to structure of murine norovirus and further resulted in the infectivity reduction. In contrast, the potential modification of MS2 bacteriophage capsid at specific sites did not substantially change the viral structure or capability of infection. Therefore, the different disinfection kinetics of two typical RNA viruses by PAA could be attributed to the sites of susceptible amino acids on viral capsids that are related with essential structures for infection. Further research is needed to confirm these findings and establish a better

understanding of viral inactivation via PAA.

Conclusions

This study examined the consumption rate of PAA by amino acids and nucleotide at a molarity ratio of 1:5 (PAA:target) in phosphate buffer (1 mM, pH 7). Range finding experiments were performed to determine the optimal concentration and pH of phosphate buffer for using the DPD method for PAA analysis. These results showed that 17 out of 21 amino acids and 4 out of 4 nucleotides did not actively react with PAA during a reaction time of 120 min. Sulfur-containing amino acids (cysteine, cystine and methionine) were proven to be the most reactive. PAA was reacted completely with cysteine and methionine in less than 2 minutes. The reactions of PAA with tryptophan and cystine followed pesudo-first-order kinetics and rate constants of PAA were determined to be 1.4×10^{-3} min^{-1} and 1.9×10^{-2} min^{-1}, respectively. It was also implicated that thiol group and thioether group were more active than disulfide bond. The same experiments on H_2O_2 demonstrated that the oxidation was mainly driven by PAA, rather than H_2O_2. Active reaction was only observed for cysteine, with consumption rate constant of 4.3×10^{-3} min^{-1}.

The low reactivity of nucleotides with PAA indicated that genome may play a less important role in PAA disinfection than capsid. Compositional data of capsids for MS2 bacteriophage and murine norovirus were employed to predict relative reaction rates of their capsids. Results showed that the abundances of susceptible amino acids and predicted reactivity of capsids for both viruses were close to each other. The resistance of MS2 bacteriophage to PAA disinfection observed in previous study could be attributed to structural components.

This study will improve diagnostics for viral inactivation using alternative disinfectants, such as PAA, which can achieve efficient microbial inactivation for water reuse at a lower cost. It can potentially further protect public health when water reuse is implemented. With an effective and economic method of disinfection, more wastewater is expected to be reclaimed and recycled.

Tables and Figures

Table 1. Concentration confirmation of disinfectants by titration

Commercial Disinfectant	Component	Measurements (g/L)
PeroxyChem PAA	PAA	213.01±2.85
	H_2O_2	265.00±1.75
Thermo Fisher H_2O_2	H_2O_2	353.75±1.79

Table 2. pH and concentration range finding for phosphate buffer

PB (mM)	min.	pH 6 (ppm)	pH 7 (ppm)
130	2	0.18±0.03	0.13±0.03
	60	0.12±0.03	0.13±0.03
100	2	3.03±0.16	3.11±0.06
	60	3.04±0.09	2.90±0.03
10	2	2.66±0.10	2.80±0.22
	60	0.85±0.16	0.75±0.04
1	2	3.03±0.19	2.99±0.19
	60	3.10±0.04	2.48±0.15
0.1	2	2.78±0.06	2.62±0.15
	60	2.62±0.03	1.98±0.07

PAA working stock was spiked into PBs of different concentrations to make sure the initial concentration to be 3 ppm. PAA solutions were maintained with gentle magnetic stirring and PAA was measured at 2 and 60 min.

Table 3. Effects of open reaction system and cations on PAA experiments

Solution	min.	Open	Sealed
10 mM PB	2	3.16±0.07	2.93±x0.07
	60	2.65±0.02	2.74±x0.02
0.1 mM PB	2	3.27±0.04	3.11±0.05
	60	3.27±0.02	3.18±0.03
10 mM NaCl	2	3.67±0.03	/
	60	3.64±0.03	/
10 mM KCl	2	3.55±0.04	/
	60	3.40±0.02	/

Open reaction system: PAA solutions maintained in glass beakers with gentle magnetic stirring;
Sealed reaction system: PAA solutions maintained in glass vials sealed with lids.

Table 4. Consumption of PAA and H_2O_2 by amino acids at 2 and 120 min

		PAA Consumed (ppm)		H_2O_2 Consumed (ppm)	
R Group	Amino Acid	2 min	120 min	2 min	120 min
Aliphatic	Glycine	-0.15±0.09 (0%)	-0.03±0.13 (0%)	0.00±0.06 (0%)	0.05±0.11 (1.3%)
	Alanine	-0.14±0.09 (0%)	0.03±0.09 (1.0%)	-0.01±0.07 (0%)	-0.02±0.05 (0%)
	Valine	-0.10±0.07 (0%)	0.11±0.13 (3.6%)	0.00±0.04 (0%)	0.00±0.03 (0%)
	Leucine	-0.12±0.09 (0%)	0.35±0.35 (11.5%)	-0.01±0.09 (0%)	-0.03±0.07 (0%)
	Isoleucine	-0.13±0.11 (0%)	0.01±0.05 (0.3%)	0.00±0.15 (0%)	-0.03±0.08 (0%)
Aromatic	Phenylalanine	-0.11±0.10 (0%)	0.27±0.45 (8.9%)	-0.02±0.08 (0%)	-0.06±0.07 (0%)
	Tyrosine	-0.14±0.10 (0%)	-0.08±0.10 (0%)	-0.02±0.06 (0%)	-0.05±0.04 (0%)
	Tryptophan	0.28±0.06 (9.2%)	0.70±0.15 (23.0%)	-0.05±0.04 (0%)	-0.05±0.10 (0%)
Hydroxyl	Serine	-0.18±0.07 (0%)	0.15±0.22 (4.9%)	0.00±0.05 (0%)	0.04±0.12 (1.1%)
	Threonine	-0.13±0.08 (0%)	0.06±0.07 (2.0%)	-0.04±0.05 (0%)	0.04±0.20 (1.1%)
Sulfur	Cysteine	>2.94±0.00 (>96.7%)	>2.94±0.00 (>96.7%)	0.10±0.03 (2.7%)	1.57±0.14 (42.8%)
	Methionine	2.74±0.01 (90.1%)	2.82±0.02 (92.8%)	-0.04±0.08 (0%)	0.04±0.12 (1.1%)
Acid	Aspartic acid*	-0.10±0.07 (0%)	0.01±0.06 (0.3%)	0.00±0.03 (0%)	-0.05±0.03 (0%)
	Asparagine	0.01±0.20 (0%)	-0.06±0.06 (0%)	-0.08±0.07 (0%)	-0.08±0.05 (0%)
	Glutamic acid*	-0.19±0.05 (0%)	-0.02±0.05 (0%)	0.00±0.02 (0%)	-0.08±0.03 (0%)
	Glutamine	-0.14±0.06 (0%)	0.08±0.18 (2.6%)	-0.02±0.06 (0%)	-0.04±0.05 (0%)
Basic	Lysine*	-0.15±0.06 (0%)	0.27±0.46 (8.9%)	0.00±0.03 (0%)	-0.02±0.03 (0%)
	Arginine*	-0.12±0.02 (0%)	0.03±0.10 (1.0%)	0.00±0.03 (0%)	-0.04±0.02 (0%)
	Histidine	-0.18±0.02 (0%)	0.29±0.60 (9.5%)	-0.04±0.03 (0%)	-0.03±0.04 (0%)
/	Proline	-0.15±0.05 (0%)	0.07±0.17 (2.3%)	-0.04±0.02 (0%)	-0.05±0.07 (0%)
/	Cystine	-0.01±0.06 (0%)	2.71±0.15 (89.1%)	-0.04±0.02 (0%)	-0.03±0.06 (0%)

* Amino acids that required extra pH adjustment in PB prior to experiments

Values were reported based on the differences between expected concentration (3.04 ppm) and measurements.

The consumption percentages shown in parenthesis were calculated based on means of measurements and were reported as 0% when negative.

Table 5. Consumption of PAA and H_2O_2 by nucleotides at 2 and 120 min

	PAA Consumed (ppm)		H_2O_2 Consumed (ppm)	
Nucleotide	2 min	120 min	2 min	120 min
AMP*	-0.11±0.07 (0%)	0.05±0.16 (1.6 %)	0.00±0.03 (0%)	-0.03±0.02 (0%)
CMP*	-0.09±0.08 (0%)	0.05±0.07 (1.6 %)	-0.02±0.03 (0%)	-0.06±0.03 (0%)
GMP	-0.16±0.10 (0%)	-0.06±0.11 (0 %)	-0.04±0.06 (0%)	-0.07±0.02 (0%)
UMP	-0.15±0.03 (0%)	-0.03±0.05 (0 %)	0.03±0.04 (0.8%)	0.01±0.01 (0.3%)

* Nucleotides that required extra pH adjustment in PB prior to experiments

Values were reported based on the differences between expected concentration (3.04 ppm) and measurements.

The consumption percentages shown in parenthesis were calculated based on means of measurements and were reported as 0% when negative.

Table 6. Abundance of selected amino acids in capsids of MS2 bacteriophage and murine norovirus

	Abundance		
Amino Acid	MS2 Bacteriophage	Murine Norovirus	Estimated Reaction Rate (min^{-1})
Cysteine	1.55%	1.32%	1.7
Methionine	1.55%	2.07%	1.2
Tryptophan	1.55%	1.32%	1.4×10^{-3}
Predicted capsid reaction rate	0.045 min^{-1}	0.047 min^{-1}	

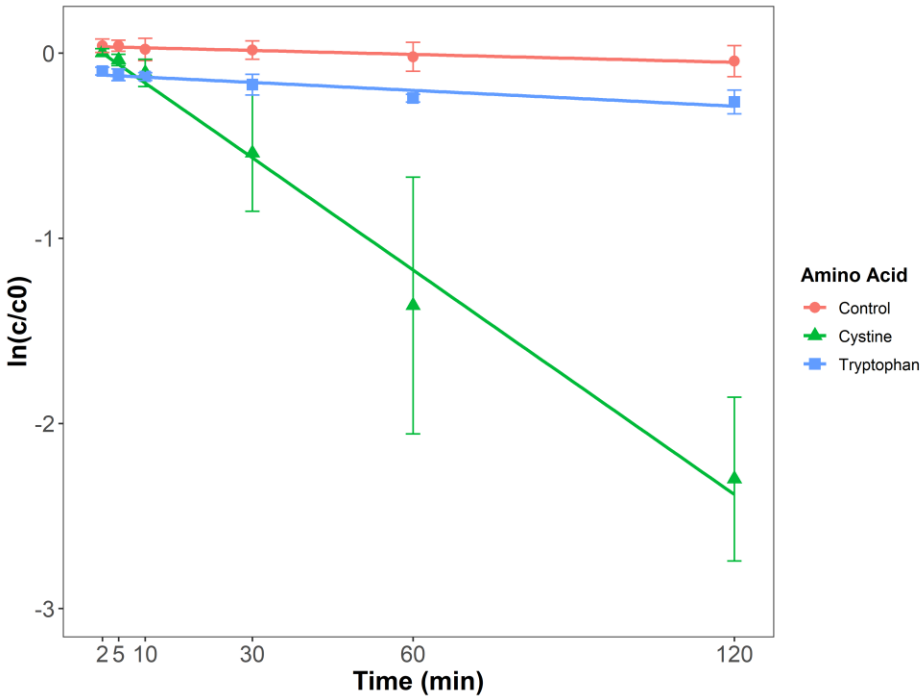

Figure 1. Consumption rate of PAA by cystine and tryptophan

Relationship between $\ln(c/c_0)$ and time for PAA consumed by control (pink circles), cystine (green triangles) and tryptophan (blue squares). Solid lines represent linear regressions of pseudo-first-order kinetics. Experiment conditions: $[\text{amino acid}]_0$=200 μM, $[\text{PAA}]_0$=40 μM, pH=7, room temperature

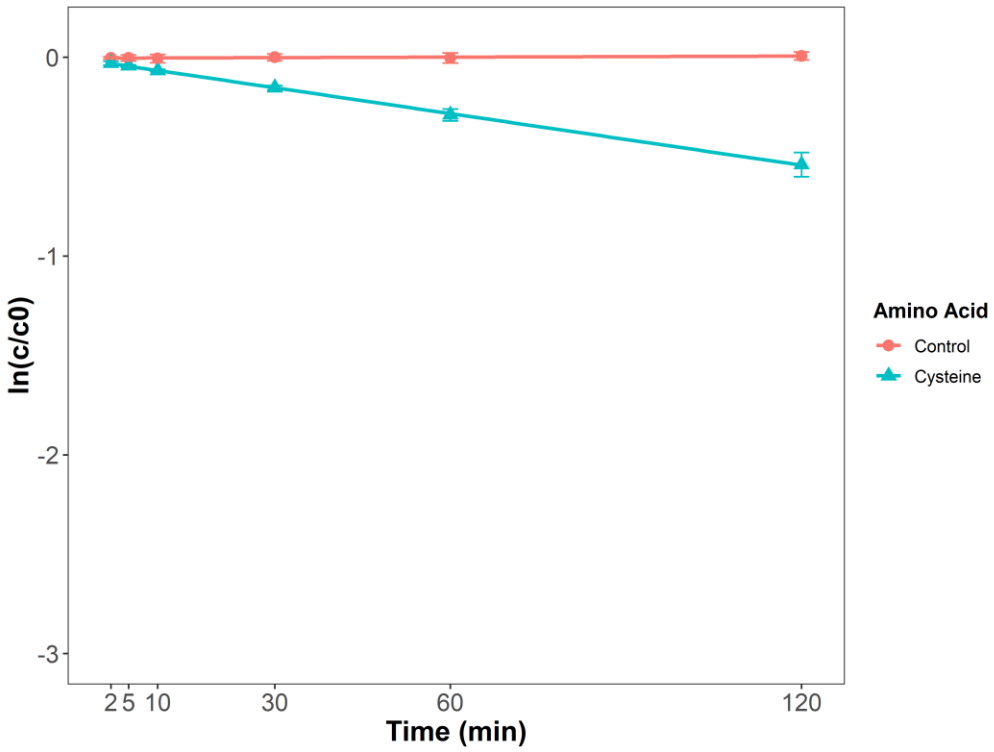

Figure 2. Consumption rate of H_2O_2 by cysteine

Relationship between $\ln(c/c_0)$ and time for H_2O_2 consumed by control (pink circles) and cysteine(blue triangles). Solid lines represent linear regressions of pseudo-first-order kinetics. Experiment conditions: $[\text{amino acid}]_0$=200 μM, $[H_2O_2]_0$=110 μM, pH=7, room temperature

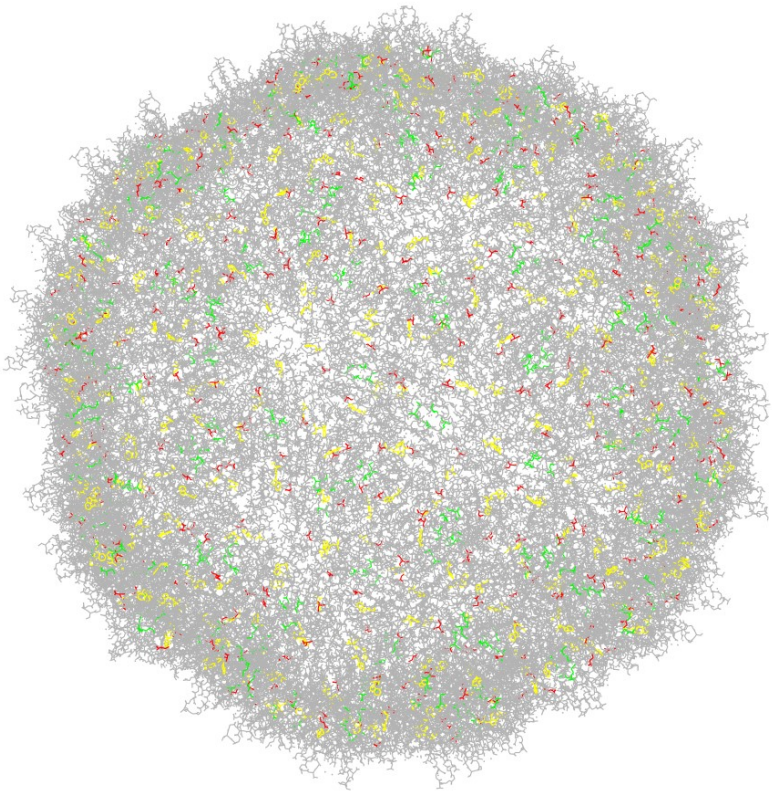

Figure 3. MS2 bacteriophage capsid

Cysteine residues are highlighted in red. Methionine residues are highlighted in green. Tryptophan residues are highlighted in yellow.

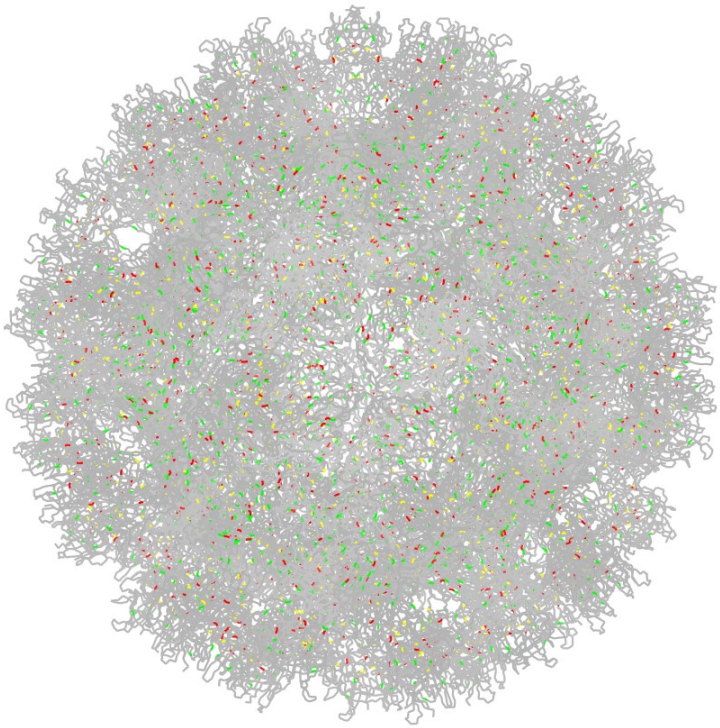

Figure 4. Murine norovirus capsid

Cysteine residues are highlighted in red. Methionine residues are highlighted in green. Tryptophan residues are highlighted in yellow

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Biography

Hanwei Wang was born in 1995 in China.

Hanwei received a Bachelor's degree in Environmental Engineering from Dalian University of Technology, Liaoning, China. During her undergraduate studies, she worked as research assistant in the State Key Laboratory of Fine Chemicals and participated in a project on synthesis and comparing properties of anionic surfactants, which was published in a peer-review journal. She also spent two years working on sampling of groundwater from rural areas of China and chemical analysis of micro-pollutants and trace elements in groundwater.

In 2017, Hanwei started her Master's degree in Environmental Health at Johns Hopkins Bloomberg School of Public Health. During her studies, she received a Certificate in Risk Sciences and Public Health, as well as a Certificate in Food Systems, the Environment and Public Health. Throughout the summer in 2018, she worked as research intern in Rwanda analyzing gender-based violence of women from refugee camps and the impact of clean cookstoves and empowerment trainings on women's health and well-being. She also worked as a teaching assistant for the course "A Built Environment and for a Healthy and Sustainable Future", assisting in promoting educational development, managing online resources and grading assignments. Upon completion of this thesis, Hanwei is expected to graduate from the Master's program in May 2019.